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Serial No.: 08/335,480 Group Art Unit: 1205

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Title: METHOD FOR TREATING ASTHMA USING OPTICALLY PURE (R)-ALBUTEROL

CERTIFICATE OF MAILING

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Respectfully submitted,

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Sheet 1 of 1

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	CB	Brittain et al. "Some observations on the β-adrenoceptor agonist..." Br. J. Pharmac. 48, 144-147 (1973)
	CC	Hartley et al. "Absolute Configuration of the Optical Isomers of Salbutamol" J. Med. Chem. 12, 995 (1991)
	CD	Hawkins et al. "Relative Potency of (-)- and (+)-Salbutamol on Guinea Pig..." J. Med. Chem. 16, 856-857 (1973)
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ANALYSIS OF SALBUTAMOL ENANTIOMERS IN HUMAN URINE BY CHIRAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PRELIMINARY STUDIES RELATED TO THE STEREOSELECTIVE DISPOSITION KINETICS IN MAN

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(First received April 8th, 1987; revised manuscript received July 7th, 1987)

SUMMARY

Enantiomers of salbutamol, extracted from human urine, were successfully separated and quantitated by high-performance liquid chromatography with electrochemical detection. This direct resolution was accomplished using a chiral α_1-acid glycoprotein column (EnantioPac) maintained at 0°C and a mobile phase consisting of a 0.1% (v/v) triethylamine in 5.3 *mM* citrate buffer, pH 7.2. An amperometric detector incorporating a glassy carbon electrode was employed for detection. The between-day coefficients of variation for the determination of *R*(−)- and *S*(+)-salbutamol in human urine were 4.1 and 4.7%, respectively, at a drug level of 1.0 μg/ml. The urinary excretion ratio of the biologically active (−)-isomer to (+)-isomer in one healthy subject who received an intravenous dose of racemic salbutamol (1.0 mg) decreased continuously over a 12-h period. A similar excretion pattern exhibiting a far more extensive distortion in the enantiomeric ratio was found in three subjects dosed with a single 4-mg tablet of racemic salbutamol.

INTRODUCTION

Salbutamol, 2-*tert*.-butylamino-1-(4-hydroxy-3-hydroxymethyl)phenylethanol, is a relatively selective β_2-adrenoreceptor stimulant widely used as a therapeutic agent in bronchial asthma and other forms of reactive airways disease [1,2]. Like many β-adrenergic agents, it is used clinically as a racemic mixture of two optical isomers, *R*(−)- and *S*(+)-salbutamol. Even though both enantiomers show high selectivity of action as the racemate for β-adrenoreceptors in tracheobronchial muscle compared to cardiac muscle [3], the drug's agonistic activity resides mainly in the *R*(−) configuration [3–5], as is the case with other β-adrenoreceptor agonists.

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Stereoselective disposition of enantiomers can result in different pharmacological profiles owing to different rates of absorption or stereoselective presystemic metabolism, distribution or clearance [6–8]. To date there is no information on the disposition kinetics of individual salbutamol isomers in man or in animals after administration of the racemic drug. The present study was undertaken to investigate, using chiral high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ED), if stereoselective disposition of *R*(−)- and *S*(+)-salbutamol exists in man after intravenous or oral dosing with the racemic drug.

The direct stereoisomeric separation and quantitation of *R*(−)- and *S*(+)-salbutamol was accomplished in this study using a chiral α_1-acid glycoprotein column which is commercially available as EnantioPac. A simple and specific urine analysis for the quantitative measurement of the enantiomers was developed based on the established separation system. The method requires sample clean-up procedures involving column extraction of the enantiomers and the internal standard followed by ion-pair extraction prior to HPLC analysis.

EXPERIMENTAL

Materials

Racemic salbutamol sulphate and terbutaline sulphate (the internal standard) were a generous gift from Glaxo Canada (Toronto, Canada) and Astra Pharmaceuticals (Mississauga, Canada), respectively. Glass-distilled ethyl acetate and HPLC-grade methanol were purchased from BDH (Toronto, Canada) and Caledon Labs. (Georgetown, Canada), respectively, and used without further purification. Di-(2-ethylhexyl) phosphate (DEHP) was a synthetic-grade reagent obtained from Sigma (St. Louis, MO, U.S.A.) and gold-label triethylamine was obtained from Aldrich (Milwaukee, WI, U.S.A.). Disposable C_8 solid-phase columns were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). The remaining chemicals and solvents were of reagent grade and were used as purchased.

Instrumentation and chromatographic conditions

The HPLC system consisted of a Beckman Model 100A pump (Berkeley, CA, U.S.A.), a Waters Model U6K injector (Milford, MA, U.S.A.), a 100×4.0 mm I.D. EnantioPac cartridge column (LKB-Produkter, Bromma, Sweden) with α_1-acid glycoprotein as the stationary phase, and an electrochemical cell containing a glassy carbon electrode controlled by a BAS Model LC-4 amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) which was connected to a Perkin-Elmer Model 024 recorder (Norwalk, CT, U.S.A.). The detector was typically operated at a sensitivity of 50 nA full scale and at an applied potential of +0.75 V vs. Ag/AgCl reference electrode. The α_1-acid glycoprotein column was embedded in an ice–water mixture in order to maintain its temperature at about 0°C throughout the analysis.

The mobile phase consisted of a 0.1% (v/v) triethylamine in 5.3 m*M* citrate buffer, pH 7.2, containing no organic modifer. It was filtered through a 0.45-μm

membrane (Millipore, Bedford, MA, U.S.A.) before use. The flow-rate was set at 0.2 ml/min.

Determination of the order of elution

Those fractions of the column eluate containing the resolved enantiomers from the chromatography of racemic salbutamol were collected and evaporated to dryness by freeze-drying. Their direction of rotation (+ or −) was determined by using a Jasco Model J-41A spectropolarimeter (Jasco Spectroscopic, Tokyo, Japan).

Sample preparation

If necessary, the pH of a 1.0-ml urine sample was adjusted to 7.2–7.5 with 0.1 *M* phosphate buffer (pH 8.8). A 30-μl internal standard solution (3 μg racemic terbutaline sulphate) was then added. The C_8 minicolumn was pretreated by successively washing it with 2 ml methanol, water and 0.1 *M* phosphate buffer (pH 7.3). The urine sample was then slowly forced through the minicolumn until the meniscus was level with the top of the column packing. The column was then washed with 2 ml of 0.1 *M* phosphate buffer (pH 7.3) and 2 ml of water. Salbutamol and the internal standard were subsequently eluted with 500 μl methanol which was evaporated to dryness under a nitrogen stream. They were further extracted as the ion pairs with DEHP by vortexing vigorously for 1 min with 50 μl of 0.1 *M* phosphate buffer (pH 7.3) and 300 μl of 0.05% (v/v) DEHP in ethyl acetate. After centrifugation the organic phase was transferred to another microtube (50×6 mm) containing 70 μl of 15 m*M* hydrochloric acid into which salbutamol and terbutaline were back-extracted by vortexing for 1 min. The organic layer was discarded after centrifugation and a small volume (1–7 μl) of the aqueous phase was injected into the chromatograph.

Sample collection

Racemic salbutamol (4.0 mg) was given as a single tablet (commercial Ventolin, A & H) to three healthy volunteers (two males and one female) between the ages of 29 and 33. One male subject also received 1.0 mg of racemic salbutamol (Ventolin) administered by intravenous infusion over a period of 30 min. Urine samples were collected without addition of any preservative at frequent intervals through 12 h, stored at 4°C and analysed in duplicate the following day.

Quantitation

Quantitation of salbutamol enantiomers was done by comparing the peak-height ratio of each enantiomer to terbutaline (T_1), the internal standard, in the unknown sample to those of control samples containing known quantities of salbutamol enantiomers, extracted and chromatographed in exactly the same way. Peak heights of salbutamol enantiomers were measured by the perpendicular distance from the peak apex to the projected baseline underneath the isomeric peaks. Readings were taken only when no shift in the zero baseline occurred, i.e. when the projected baseline was horizontal.

RESULTS AND DISCUSSION

Enantiomeric order of elution

The assignment of a + or − sign to the resolved enantiomers are in accordance with their Cotton effect at 276–280 nm as measured on the Jasco spectropolarimeter. Thus, under the established chromatographic conditions, *S*(+)-salbutamol was found to elute faster than *R*(−)-salbutamol from the chiral column. The order of elution of terbutaline enantiomers (the internal standard) was not determined. Therefore, T_1 and T_2 were assigned to the enantiomer with the shorter and longer elution time, respectively.

Enantioseparation studies

The most challenging task during the course of this study undoubtedly was to achieve a chromatographic separation of the stereoisomers of salbutamol. Our attempts to explore both the direct and indirect chromatographic resolution methods were partially limited by the requirements of an HPLC system compatible with ED. Our experiments to derivatise salbutamol with some commonly used chiral derivatising agents followed by diastereomeric resolution on a reversed-phase column were not successful. Additionally, direct resolution employing a reversed-phase mode of operation on several types of chiral stationary phase, such as D-phenylglycine or L-leucine (Pirkle covalent column), β-cyclodextrin (Cyclobond I column) and cellulose ester (Chiralcel Type OK column), also failed to resolve the enantiomers of salbutamol. Visibly discernible separation was first realised with the α_1-acid glycoprotein column (EnantioPac) at room temperature using a buffered aqueous mobile phase for elution. This separation was dramatically enhanced upon lowering the column temperature. The lack of a cooling device to maintain the column temperature at the recommended minimum of 4 °C prompted us to embed the column in an ice–water mixture. No serious deterioration in column performance was observed. Organic modifier was found to diminish both the retention and selectivity, and hence was excluded from the mobile phase. Higher ionic strength reduced selectivity and therefore the molarity of the buffer was kept low though high enough to maintain sufficient buffering capacity and current conduction. The pH of the buffer was also important as the capacity and resolution factor increased with an increase in pH, as expected for cationic substrates. A pH of 7.2 was chosen to avoid shortening column life. The type of buffer used was not critical but citrate buffer was chosen over acetate or phosphate buffer as it was observed to be significantly better in terms of selectivity. The addition of a tertiary amine, triethylamine, to the mobile phase followed by titration to the appropriate pH further improved the selectivity with optimal resolution being obtained at 0.1% (v/v) triethylamine. The final composition of the mobile phase was established through the extensive manipulations of these parameters until the best possible resolution was obtained, as shown in Fig. 1. The relatively low retention capacity ($1.0 < k' < 2.0$) of the column for salbutamol does not permit a total baseline separation but the resolution ($R_s = 1.06$) achieved here is believed to be adequate to allow good kinetic data to be obtained unless the concentration ratio of the enantiomers is $> 10:1$.

Fig. 1. Enantiomeric separation of 40 ng (−)- and (+)-salbutamol on the EnantioPac column. Chromatographic data: $k'_{1,-}=1.22$; $k'_{1,-}=1.55$; $\alpha=1.27$; $R_s=1.08$. Peaks (+) = S(+)-salbutamol (−) = R(−)-salbutamol.

Urinary assay

The enantiomeric separation system established above was then used for the quantitative determination of the enantiomeric composition of salbutamol in human urine. Fig. 2 represents typical chromatograms of extracted blank urine (A) and of a urine sample (B) to which 2 μg/ml racemic salbutamol had been added. No interfering peaks were observed in several different pools of blank urine when

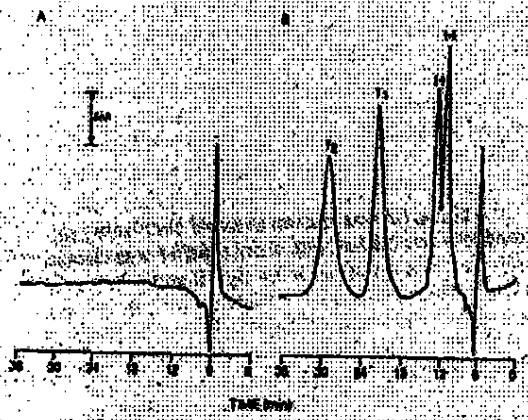

Fig. 2. Chromatograms obtained with (A) blank urine and (B) urine containing 2 μg/ml racemic salbutamol. Peaks: (+) = S(+)-salbutamol; (−) = R(−)-salbutamol; T_4, T_5 = (±)-terbutaline, the internal standard.

TABLE I

PRECISION AND RECOVERY DATA FOR ENANTIOMERIC SALBUTAMOL ASSAY

C.V. = coefficient of variation.

Racemic salbutamol concentration (μg/ml)	Precision ($n = 8$)				Recovery (mean ± S.D., $n = 7$) (%)	
	Within-day C.V. (%)		Between-day C.V. (%)			
	(+)	(−)	(+)	(−)	(+)	(−)
0.50	3.5	3.9	4.8	4.4		
2.00			4.7	4.1	57.4 ± 8.3	57.0 ± 8.5
5.00	3.0	3.8	4.2	4.0		

the sample injection volume was kept small. The resolution of salbutamol enantiomers in extracted urine samples appears to be significantly less than that of aqueous standards, presumably due to the medium in which salbutamol was injected into the chromatograph (hydrochloric acid versus water). Note that racemic terbutaline, a β_2-adrenoceptor agonist closely related to salbutamol and used as the internal standard in this analysis, is very well separated from both salbutamol enantiomers. The terbutaline enantiomer with the lower retention capacity, T_1, was used for calculation throughout the study.

Assay validation

The analytical recovery of salbutamol enantiomers in seven urine samples spiked with 2.0 μg/ml was essentially identical for both enantiomers, averaging 57% (Table I). The precision of the method was assessed by the repeated analysis of urine samples to which racemic salbutamol had been added at different concentrations ranging from 0.50 to 5.0 μg/ml. The coefficients of variation for both the within-day and between-day assays are summarized in Table I.

The standard curves of the two salbutamol isomers were constructed as the peak-height ratios of $R(-)$- or $S(+)$-salbutamol to terbutaline (T_1) versus concentrations of the salbutamol enantiomer. Concentration and peak-height ratio were verified to be linearly related throughout the concentration range examined (0.25–5.0 μg/ml), yielding a correlation coefficient of 0.9985 for each enantiomer.

Chromatographic analysis time for a urine sample requires 30–35 min. Specificity of the assay is good. In no instance did the pre-trial sample yield peaks with the same retention time as salbutamol or the internal standard. The sensitivity is limited by the small injection volume used (<10 μl).

Salbutamol enantiomers in human urine

Timed urine samples collected from the subject who received intravenous racemic salbutamol showed a continuously decreasing proportion of the active $R(-)$-isomer to the relatively inactive $S(+)$-isomer. The excretion ratio of (−)- to (+)-salbutamol obtained 1 h after drug infusion was 0.89 but declined to 0.59 for the 8–12 h collection period. On the other hand, urine specimens collected on

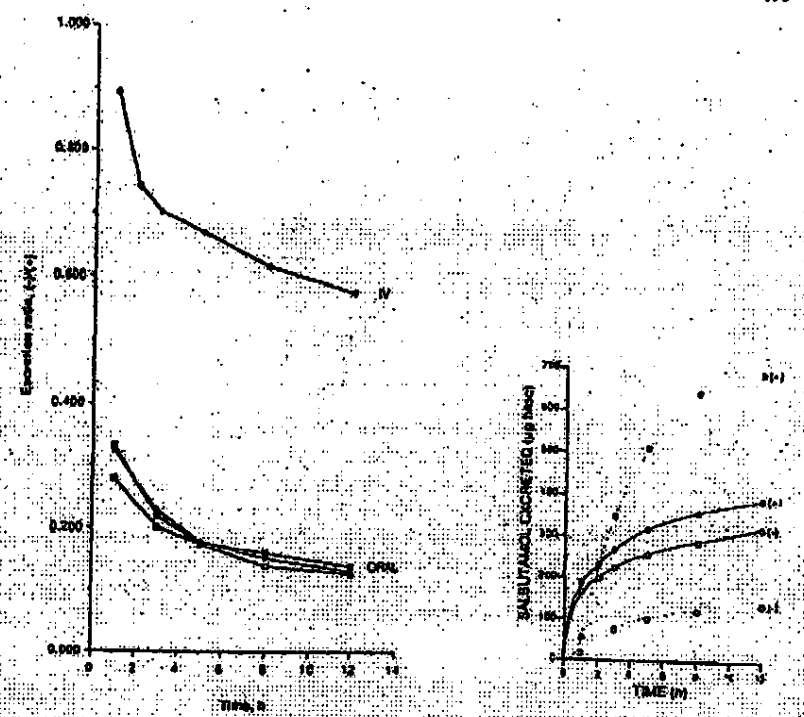

Fig. 3. Decreasing excretion ratio of the active (−)-salbutamol in urine collected up to 12 h after the oral ingestion and intravenous infusion of 4.0 and 1.0 mg of (±)-salbutamol, respectively, in a total of four experiments.

Fig. 4. Cumulative urinary excretion of *S*(+)-salbutamol (●) and *R*(−)-salbutamol (○) through 12 h from one male subject (age 28 years, weight 50 kg) who received oral (---) and intravenous (——) administration of 4.0 and 1.0 mg of racemic salbutamol, respectively, on two separate occasions.

the same subject plus two others after oral ingestion revealed a similar pattern of excretion but an even more extensive distortion in the enantiomeric ratio was observed, as shown in Fig. 3. The extent of such distortion remained relatively constant over a 12-h period for the three subjects studied. Fig. 4 shows the 12-h cumulative amount of the isomers excreted in the one subject who was dosed with both intravenous and oral racemic salbutamol on two separate occasions.

The findings above clearly indicate that the renal excretion rates of the two parent (unmetabolized) enantiomers are not the same and are dependent on the route of administration. As salbutamol is known to undergo extensive presystemic extraction in man following oral administration [9,10], the present work suggests strongly the existence of a stereoselective first-pass metabolism with the more potent *R*(−)-isomer being preferentially metabolised, resulting in a re-

TABLE II

EFFECTS OF ARYLSULFATASE HYDROLYSIS OF EXTRACTED URINE SAMPLES (0–5 h) ON SALBUTAMOL ENANTIOMERIC RATIO

Measured in terms of chromatographic peak heights. Unless otherwise stated, the hydrolytic conditions were: incubation volume, 0.5 ml; incubation time, 15 h; incubation medium, 0.1 *M* acetate buffer at pH 5.0; amount of enzyme, 2200 U.

Enzyme quantity		Incubation time		Incubation medium	
Unit (U)	(−)/(+) ratio	Time (h)	(−)/(+) ratio	Medium, pH 5.0	(−)/(+) ratio
0	0.21	2	0.45	Urine	0.35
80	0.26	15	1.00	Phosphate buffer	0.32
2200	1.00	29	1.05	Acetate buffer	1.00
6400	1.07	40	1.08		

duced systemic availability of the active drug. Such preferential elimination of the more potent isomer during first-pass metabolism is well documented with the calcium antagonist verapamil, and explains the apparent paradox that following oral ingestion, two to three times higher plasma verapamil concentrations in comparison to intravenous administration were required to produce an equivalent dromotropic effect on atrioventricular conduction in man [11]. The major metabolite of salbutamol in human urine has been isolated and identified to be the 4′-O-sulphate ester [12,13]. Morgan et al. [14] have recently suggested that the first-pass elimination of salbutamol is due entirely to sulphate conjugation. In our hands, enzymatic hydrolysis at pH 5.0 with arylsulfatase of *Helix pomatia* of this pharmacologically inactive conjugate, which was extracted from the 0–5 h urine samples collected after oral ingestion using C_{18} minicolumns (Waters Assoc., Milford, MA, U.S.A.), increased the (−)- to (+)-salbutamol ratio to near or above unity, thus confirming that the former isomer is preferentially conjugated with sulphate. As phosphate ion is known to inhibit the arylsulfatase activity of *Helix pomatia* [12], neither incubation of the sulfatase directly in urine sample (which contains phosphate ions) nor incubation with the extracted urine sample in phosphate buffer produced any significant increase in the amount of the free (−)-salbutamol (Table II). It thus appears that stereoselective sulphate conjugation is the most predominant mechanism responsible for the differential disposition kinetics of (+)- and (−)-salbutamol seen in man, particularly after oral administration.

CONCLUSIONS

This paper describes a chiral HPLC separation method for the optical isomers of salbutamol, a bronchodilatory drug widely used in asthma therapy around the world. The established separation technique was employed to quantitatively determine the two salbutamol enantiomers extracted from human urine after the oral or intravenous administration of the racemic drug. The resolution achieved

with the chiral α_1-acid glycoprotein column has allowed sufficiently good pharmacokinetic data to be derived until the concentration difference between the isomers becomes substantial. Preliminary results strongly favour the existence of an enantioselective mechanism(s) that results in the preferential removal of the active *R*(−)-salbutamol from the body particularly after oral ingestion of the racemate. Enzymatic hydrolysis studies point to the first-pass biotransformation, specifically the 4′-*O*-sulphate conjugation, as the dominant mechanism responsible for the differential disposition of the enantiomers seen in man after oral ingestion. In light of the present findings, it appears important to study concentration–effect relationships of salbutamol, and possibly of other β-adrenoceptor agonists, based on the measurement of the active isomers. To quote Ariëns [15], the neglect of the stereoselectivity in drug action degrades many otherwise good pharmacokinetic studies to expensive "highly sophisticated pseudoscientific nonsense".

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Some observations on the β-adrenoceptor agonist properties of the isomers of salbutamol

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Summary

1. The pharmacological activities of the optical isomers of salbutamol have been examined. (−)-Salbutamol was much more potent than (+)-salbutamol on β-adrenoceptors.

2. Both (−)- and (+)-salbutamol showed high selectivity for β-adrenoceptors in bronchial muscle compared to cardiac muscle, in this way resembling racemic salbutamol.

3. The use of isomeric activity ratio to detect differences between receptors was examined in the light of the results obtained with the isomers of salbutamol.

Introduction

Lands and his colleagues (Lands, Arnold, McAuliff, Luduena & Brown, 1967; Lands, Luduena & Buzzo, 1967) proposed that β-adrenoceptors be classified into β_1 and β_2 types. Stimulation of mammalian cardiac muscle is mediated by β_1-receptors and relaxation of bronchial, arterial and uterine muscle by β_2-receptors. Skeletal muscle also contains β_2-receptors. This classification, which was based on the relative potencies of N- and α-substituted catecholamines in different tissues, has gained more general acceptance since the discovery of highly selective β-adrenoceptor agonists (see Brittain, Jack & Ritchie, 1970). Salbutamol is a β-adrenoceptor agonist which is more active on bronchial smooth muscle than on cardiac muscle (Cullum, Farmer, Jack & Levy, 1969; Daly, Farmer & Levy, 1971). This drug contains an asymmetric centre and so it was of interest to ascertain whether activity resided mainly in the laevo (−) isomer (R configuration), as in the case with other sympathomimetic amines acting on adrenoceptors, and whether the isomers showed the same selectivity of action as the racemate. Recently Hartley & Middlemiss (1971) prepared the (−) and (+) isomers of salbutamol and this paper describes some pharmacological properties of these compounds.

Methods

Guinea-pigs of either sex, weighing 250–400 g were anaesthetized with urethane, 1·25 g/kg i.p., and prepared for measurement of bronchial resistance (Konzett & Rössler, 1940). Temporary increases in bronchial resistance, measured with a low pressure transducer connected to a Devices recorder, were produced by sub-maximal doses of acetylcholine, histamine or 5-hydroxytryptamine injected intravenously at intervals of 5 minutes. β-Adrenoceptor agonists were injected intravenously 5 min before intravenous injection of the spasmogens.

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of either sex, weighing 7–12 kg, were anaesthetized with pento-
m, 30 mg/kg intravenously. The animals were intubated with a
heal tube and allowed to breathe spontaneously, except in those
olving pulmonary resistance tests. Arterial blood pressure was
carotid artery or femoral artery by means of a Bell & Howell blood
ucer connected to a Devices recorder. Heart rate was measured
instantaneous rate meter triggered by the pulse pressure. Measure-
ary air flow, pressure and volume were made as described by
. Pulmonary resistance was calculated by the method of Amdur &
temporary changes in pulmonary resistance were induced by intra-
of acetylcholine at 5 min intervals. β-Adrenoceptor agonists were
ore the injection of acetylcholine. In all experiments drugs were
ously through a cannula in a femoral vein.

oncentration-effect curves for the β-adrenoceptor agonists were
he reduction of induced tone in the isolated intact trachea prepara-
a-pig (Farmer & Coleman, 1970) and for their positive inotropic
effects on isolated left and right atrial strips of the guinea-pig
adding geometrically increasing doses of drug to the tissue bath
the bathing fluid. The relative activities of the β-adrenoceptor
ressed as the doses equipotent with isoprenaline (=1) at 50%
effect.

drugs were used: (±)-isoprenaline sulphate, (±)-salbutamol hydro-
nd (+)-salbutamol acetate monomethanolate, pronethalol hydro-
opranolol hydrochloride. (±)-Isoprenaline was dissolved in 0·9%
ion (saline) containing ascorbic acid, 1 μg/ml. All other drugs
saline. In the text, concentrations refer to the free base; (±)-
ferred to as isoprenaline.

ts on bronchospasm in the anaesthetized guinea-pig

nd (−)-, (+)- and (±)-salbutamol given intravenously inhibited
ced bronchospasm in anaesthetized guinea-pigs. The dose-effect
mers and (±)-salbutamol were similar in slope and maxima to
th isoprenaline. Effective intravenous doses were found in the
/kg for isoprenaline, 2·5–100 μg/kg for (−)- and (±)-salbutamol
kg for (+)-salbutamol. The mean equipotent doses for (−)-,
lbutamol compared to isoprenaline (=1) were 2·93 (1·30–
0) and 3·75 (1·88–7·75) respectively. Similar orders of potencies
hen the spasmogen used was histamine or 5-hydroxytryptamine.
)- and (+)-salbutamol were mediated through β-adrenoceptors
e prevented by a prior injection of pronethalol, a β-adrenoceptor

cetylcholine-enhanced pulmonary resistance and on blood
ressure and heart rate in the anaesthetized dog

(−)-, (+)- and (±)-salbutamol caused dose-dependent inhibition
lmonary resistance induced by intravenous acetylcholine. The

effective intravenous dose-ranges were isoprenaline 0·1–2·0 μg/kg, (−)-salbutamol 1–5 μg/kg, (+)-salbutamol 50–400 μg/kg and (±)-salbutamol 4–20 μg/kg. The mean equipotent doses of (−)-, (+)- and (±)-salbutamol compared to isoprenaline (=1) were 2·6 (0·5–13·9), 138 (59–322) and 6·0 (3·0–12·2) respectively. Isoprenaline, (±)-salbutamol and the isomers, at the dose-ranges quoted, also caused falls in diastolic blood pressure (5–50 mmHg) but only isoprenaline caused significant increases in heart rate (10–60 beats/minute). Indeed very large doses of (−)- and (±)-salbutamol caused only small increases in heart rate; for example, the tachycardia after 100 μg/kg of either drug was only 20–25 beats/minute. (+)-Salbutamol, 400 μg/kg, had no significant effect on heart rate.

Effects on isolated tissue preparations

The effects of isoprenaline and (−)-, (+)- and (±)-salbutamol on isolated tracheal and atrial preparations of the guinea-pig are summarized in Table 1.

TABLE 1. *β-Adrenoceptor agonist activities of isoprenaline, (−)-, (+)- and (±)-salbutamol on isolated tissue preparations of the guinea-pig.*

Preparation	Receptor type (Lands *et al.*, 1967)	β-Adrenoceptor agonist potency (mean equipotent doses* relevant to isoprenaline = 1)			
		Isoprenaline	(−)-Salbutamol	(+)-Salbutamol	(±)-Salbutamol
Intact trachea	β_2	1	6·2 (3·2–11·9)	425 (313–522)	>6 (3·2–9·4)
Atria (left)	β_1	1	>10,000†	v. weak negative inotropic response	>10,000†
Atria (right)	β_1	1	>10,000†	>10,000†	≤1,000†

* Calculated on weight/volume basis. † Partial agonist.

On the intact guinea-pig trachea the β-stimulants caused dose-dependent decreases in the rise of intraluminal pressure induced by transmural stimulation. The effective concentrations were: isoprenaline 5–50 ng/ml (12·9–129 nM), (−)- and (±)-salbutamol 20–300 ng/ml (84–1,260 nM) and (+)-salbutamol 1–25 μg/ml (4·20–105 μM). The concentration-effect curves for the isomers and (±)-salbutamol were similar in slope and maxima to those obtained with isoprenaline. The drug effects were antagonized by propranolol (100 ng/ml). The actions of (−)-, (+)- and (±)-salbutamol on isolated atrial preparations of the guinea-pig were quantitatively and qualitatively different from those of isoprenaline. Isoprenaline, 0·1–5 ng/ml (0·24–12·0 nM) caused dose-dependent positive chronotropic effects on cardiac muscle whereas concentrations of 0·2–20 μg/ml (0·84–84 μM) of (−)-, (+)- and (±)-salbutamol were required to elicit a positive chronotropic effect; even then the maximum responses to salbutamol were not more than 30% of those obtained with isoprenaline. In their inotropic actions (−)- and (±)-salbutamol were weak partial agonists; surprisingly (+)-salbutamol, 10–40 μg/ml (42–168 μM), caused a very weak negative inotropic effect (5–20%).

Discussion

Detailed studies with the isomers of isoprenaline (Beccari Berethar & Lavendaio 1953) showed that the pharmacological activity of the racemate resides mainly in the (−) isomer. It was not surprising therefore to find that (−)-salbutamol was

...active than (+)-salbutamol. More interesting was the fact that both ...bined the racemic compound in being very much more active on ...th muscle than on cardiac muscle. This result makes it unnecessary ... the very relative inactivity of racemic salbutamol on cardiac muscle ... an interaction between the isomers in the tissue.

...It is argued that if β-adrenoceptors are dissimilar, then the ratios of ...the optical isomers of a β-adrenoceptor agonist in different tissues con... should be different. Buckner & Patil (1971) determined iso... ratios of (−)- and (+)-isoprenaline in isolated atrial and tracheal ... of the guinea-pig and concluded that the β-adrenoceptors in these ... are different. While the isomeric activity ratio of (−)- and (+)-salbu... in tracheal muscle is 1:68 it is impossible to calculate a ratio on ... because the isomers are virtually inactive on this tissue. As dis... (Brittain *et al.*, 1970) the selectivity of action of salbutamol ... on the nature of the *N*-substituent and the 3-substituent in the ... and not the configuration about the asymmetric carbon. The results pre... paper are in accord with Lands' proposals that β-adrenoceptors in ... muscle can be differentiated from those in cardiac muscle.

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(*Received December 18, 1972*)

...amine and ethyl acrylate using a 2-step procedure ...Balyard and McElvain[1] for the synthesis of 1-...pidone. The intermediate N,N-bis(2-carbethoxyethyl)... ...ethoxyethylamine boiled at 111° (0.05 mm). The ...lic cyclization and decarboxylation were done con-...ment solution of the intermediate 3-carbethoxy-1-...yl-4-piperidone. The final product, a colorless ...bp 72-73° (0.5 mm). Anal. ($C_{..}H_{..}NO_2$) C, H, N.

...yl-4-piperidylamino)alkyl]benzyl Alcohols (3-11). ...25 mole) was added in small portions over 2 hr to ...α-(1-aminoalkyl)-4-hydroxybenzyl alcohol ...ole), KOH (0.025 mole), and 1-alkyl-4-piperidone ...100 ml of MeOH. The reaction mixt was main-...5° by means of an ice bath during the addn. ...he mixt was stirred for 1 hr at 20° and then acidi-...with 4 N HCl in EtOH. The mixt was filtered to ...ing salts and then evapd to an oil. The residual ...ed with boiling i-PrOH. The resulting white ...iltered off and recrystd (see Table I).

...peridylamino)alkyl]benzyl Alcohols (12 and 13).— ...(benzyl-4-piperidylamino)methyl]benzyl alcohol ...(0.083 mole) in 125 ml of aq 80% EtOH was ...d 6 hr at 3 atm using 5 g of 10% Pd/C as catalyst. ...was removed by filtration, and the filtrate was ...due was recrystd (Table I).

...Assay Method.—The test substances were ...etized dogs on the vascular bed supplied by the ...In this prepn a constant flow (peristaltic) blood ...posed between the proximal and distal segments of ...al artery. Prior to drug treatment blood flow ...result in a mean perfusion pressure approximately ...the arterial blood pressure. Perfusion pressure ...distal to the pump. Drug injections were made ...tream distal to the pump. Vasodilation was ...decrease in perfusion pressure. Potency com-...apaverine were determined from plots of log ...decrease in perfusion pressure. In this assay isox-...ximately 10 times more active than papaverine. ...ted in Table I are based upon the arbitrary ...averine potency at 100.

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...figuration of the Optical Isomers of Salbutamol

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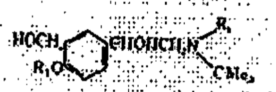
...earch, Allen and Hanburys Ltd., Ware, Hertfordshire, England

Received March 8, 1971

...and biological properties of the β-...amine, salbutamol (4), have been ...compound is more selective for β_2 re-...ously known drugs and therefore it ...tent each enantiomer to ascertain if ...mainly in the (R)-(−) configuration ...her drugs which act at adrenoceceptors.

...tions of salbutamol, or any phenolic ...uccessful.[3] However, when the phe-...intermediate ester 1 was protected ...2 then resolution was efficiently ...(+)- or (−)-di-p-toluoyltartaric ...only one isomer formed a crystalline

1, R = H
2, R = $PhCH_2$



3, R_1 = $PhCH_2$
4, R_1 = H

salt and the antipode was recovered from the mother liquors in good yield and reasonable optical purity.

Neutralization of the purified salts liberated the resolved bases 2 which on reduction with LAH followed by catalytic debenzylation gave the required isomers 4. The (+) and (−) forms were both characterized as their acetate salts.

The β-adrenoreceptive activities of these isomers were compared with those of the racemic compound on the β_1 receptors[4] of the isolated atria of the guinea pig and on the β_2 receptors[5] of the intact trachea of the guinea pig (Table I). In the latter test[5] the (−) isomer was approximately equieffective with the racemate and 80 times more potent than the (+) isomer. A similar pattern was shown in the much weaker effects on the force of contraction of the electrically driven left atrium.[6] However, both isomers were much less active than the racemate in increasing the rate of contraction of the spontaneously beating right atrium.[7] Although this result has been verified on several occasions, the very low order of activity precludes any useful interpretation of the apparent synergism in the racemate.[8]

TABLE I
BIOLOGICAL ACTIVITY OF THE ENANTIOMERS OF SALBUTAMOL

Compound	Trachea (5)	Left atrium (6)	Right atrium (6)
	Biological test[a]		
Racemate	4.3	16,000[b]	1,000
(−) Isomer	9.8	15,000[b]	10,000[b]
(+) Isomer	423	Inactive	100,000[b]

[a] These results represent the ratio of the amount of drug required to produce an equivalent response to a unit of isoprenaline. [b] Denotes partial agonist activity.

Comparison of the CD spectra of the salbutamol isomers with that of (R)-(−)-octopamine[9] showed that (−)-salbutamol had the R configuration. Both levorotatory compounds showed a clear negative Cotton effect at 270–280 nm. At lower wavelengths, 220–230 nm, the curves tended toward a further negative peak although this is somewhat masked by the high aromatic absorption.

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CD Notes

envelope and OH), 3.75 (s over a multiplet, 2, $ArCH_2$), 7.47 (s, 5, aromatic). The hydrochloride was prepared in the normal manner and recrystallized from EtOH–Et_2O, mp 203–205°. *Anal.* ($C_{13}H_{19}ClNO$) C, H, N.

Further elution with petroleum ether–Et_2O (1:1) afforded the trans alcohol (0.3 g): mp 75–76°; ir (0.002 *M* CCl_4) 3620 cm^{-1} (free OH); nmr ($CDCl_3$) δ 1.1–2.2 (broad signals, 6, bicyclic envelope), 2.2–3.0 (broad signals, 4, H-1, H-3, and H-4), 3.75 (s, 2, NCH_2Ar), 4.0–4.4 (m, 1, H-6), 7.45 (s, 5, aromatic). *Anal.* ($C_{13}H_{17}NO$) C, H, N.

6-trans-Hydroxy-2-azabicyclo[2.2.2]octane. A solution of 2-benzyl-6-*trans*-hydroxy-2-azabicyclo[2.2.2]octane (13.0 g, 0.06 mol) in EtOH (100 ml) was hydrogenated (3.15 kg/cm^2) over 10% Pd/C (1.5 g) for 24 hr. The catalyst was removed by filtration and the solvent evaporated to yield a white solid. Recrystallization from $CHCl_3$–hexane gave 6.0 g (78%) of a white solid: mp 229–231°; ir (1% $CHCl_3$) 3620 (OH), 3350 cm^{-1} (NH); nmr (DMSO-d_6) δ 1.0–2.4 (broad signals, 9, bicyclic envelope), 2.5–2.75 (m, 1, H-1), 2.8 (broad singlet, 1, NH), 3.4 (broad singlet, 1, OH), 3.75–4.2 (m, 1, H-6). *Anal.* ($C_7H_{13}NO$) C, H, N.

2-Methyl-6-*trans*-hydroxy-2-azabicyclo[2.2.2]octane (7). A solution of 6-*trans*-hydroxy-2-azabicyclo[2.2.2]octane (5.0 g, 0.04 mol) and CH_2O (5 ml of 37%) in EtOH (70 ml) was hydrogenated (3.15 kg/cm^2) over 10% Pd/C (0.3 g) for 12 hr. The catalyst was removed by filtration and the solvent evaporated to yield a yellow oil which upon distillation gave 7 (4.5 g, 80%): bp 125–128° (20 mm); picrate mp 230–231°; ir (CCl_4, 0.002 *m*) 3640 cm^{-1}. This alcohol is identical with the alcohol obtained in Scheme II.

5-cis-Hydroxy-2-azabicyclo[2.2.2]octane. A solution of 2-benzyl-5-cis-hydroxy-2-azabicyclo[2.2.2]octane (1.7 g, 0.008 mol) in EtOH (80 ml) was hydrogenated (3.15 kg/cm^2) over 10% Pd/C (0.2 g) for 3 hr. The catalyst was removed by filtration and the EtOH evaporated. The crude product was recrystallized from Et_2O to yield a white solid (0.5 g, 50%): mp 193–195° dec; ir (1% $CHCl_3$) 3645, 3620, and 3380 cm^{-1} (OH and NH).

2-Methyl-5-cis-hydroxy-2-azabicyclo[2.2.2]octane (5). A solution of 5-cis-hydroxy-2-azabicyclo[2.2.2]octane (1.0 g, 0.008 mol) and CH_2O (1 ml of 37%) in EtOH (50 ml) was hydrogenated (3.15 kg/cm^2) over 10% Pd/C (0.2 g) for 6 hr. The catalyst was removed by filtration and the EtOH evaporated. The residue was distilled to give a clear oil (0.7 g, 63%): bp 106–110° (20 mm); ir (0.002 *M* CCl_4) 3450 cm^{-1} (associated OH); picrate mp 259–260°.

General Procedure for the Synthesis of *p*-Aminobenzoate Esters 1–4. A solution of the amino alcohol (0.014 mol) and TEA (0.021 mol) in 60 ml of C_6H_6 was added dropwise to a cooled solution of *p*-nitrobenzoyl chloride (0.014 mol). The mixture was refluxed for 24 hr, cooled, and extracted with 10% HCl (3 × 50 ml). The acid extracts were combined, made basic with K_2CO_3, and extracted with $CHCl_3$ (3 × 50 ml). The $CHCl_3$ was combined, dried ($MgSO_4$), and evaporated to yield a solid which was taken up in 100 ml of EtOH and added to a Parr flask. The solution was hydrogenated (3.15 kg/cm^2) over 0.2 g of 10% Pd/C for 12 hr and filtered through Celite and the solvent was evaporated to yield an orange solid. The solid was recrystallized from the indicated solvent (Table I) to yield the desired *p*-aminobenzoate ester.

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Notes

Relative Potency of (−)- and (±)-Salbutamol on Guinea Pig Tracheal Tissue†

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The two enantiomers of the various asymmetric β-sympathomimetic drugs are usually found to have significantly different potencies. Studies with guinea pig tracheal tissue have shown that, where the absolute configuration is known, the *R* isomer is the more active and the racemate's activity lies between those of the two enantiomers. Recently, however, it was reported that racemic salbutamol (I) was 1.5 times as active as the more active (laevo) of the two enantiomers.[1,2]

I

This result is unique for this type of drug interaction and warranted further investigation especially as salbutamol's marked β_2 selectivity[3] has made it an important bronchodilator for the treatment of asthma.

This paper describes the results of relaxation studies with the isomers of salbutamol using guinea pig tracheal chains. Each tissue was tested by cumulative drug–response tests using adrenaline prior to study with salbutamol. The results are presented in Figure 1. The mean log ED_{50} values with their associated standard errors are as follows: isomer with $[\alpha]^{20}D$ −32.2°; −7.3 ± 0.06 (96.9 ± 4.2); (±), −7.61 ± 0.04 (101.2 ± 5.5); isomer with $[\alpha]^{20}D$ +30.8°, −7.50 ± 0.03 (98.9 ± 3.7). The mean slopes of the log dose–response curves with their standard errors are presented in parentheses. As the (+) isomer was not fully resolved, it would have somewhat less activity than that indicated by the above ED_{50}

†This investigation was supported by the Asthma Foundation of Queensland and the Australian Research Grants Committee.

‡Hartley and Middleniss in the text of their paper[3] considered the two to be approximately equiactive.

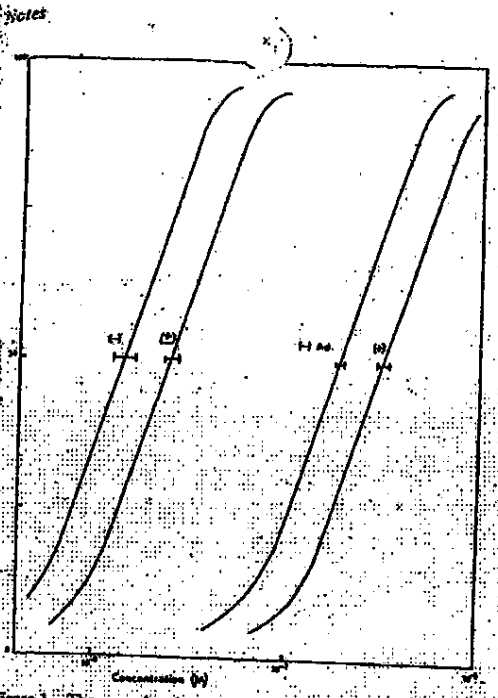

Figure 1. The mean log dose-response lines for (−), (±), and (+)-salbutamol and (−)-adrenaline. Doses are given as molar concentrations in the tissue bath. The error bars represent the standard error of the mean log ED_{50}'s.

value due to contributions from the more active (−) isomer. These results, in contrast to those of Hartley and Middlemiss,[1] show that the (−) isomer is significantly more active than the racemic in agreement with the general finding that a racemic drug's activity lies between those of the two enantiomers.[3,4] From a comparison of the response curves for a set of tissues, it was found that (−)- and (±)-salbutamol are 12.6 and 5.6 times, respectively, more active than (−)-adrenaline.

The technique used in the present investigation, which is a standard method for studying the relaxation of smooth muscle,[5] differs from that used by Hartley and Middlemiss.[1] Their method, which was developed in their own laboratory,[6] is an intraluminal pressure technique. The two techniques and preparations might be expected to give small differences in the absolute values of the ED_{50}'s but the different techniques should not yield such large variations in the relative potencies of the isomers as observed.

Experimental Section

Melting points were observed on a Büchi oil-bath melting point apparatus and microchemical analyses were performed by the Australian Microanalytical Service, Melbourne, Australia. Optical rotations were measured with a Perkin-Elmer Model 141 automatic polarimeter in H_2O at 20°. The compounds gave satisfactory uv and ir spectral data obtained with a Cary 14 and a Perkin-Elmer 225 instrument, respectively.

Resolution of 2-*tert*-Butylamino-1-(4'-hydroxy-3'-hydroxymethylphenyl)ethanol (Salbutamol). To a warm solution of racemic salbutamol[§] (0.8 g, 0.0034 mol) in dilute H_2SO_4 (4 ml) was added $(+)_{546}$-Ba[CoEDTA)$_2$·$4H_2O$ (0.76 g, 0.0008 mol), $[\alpha]^{20}_{546}$ +890° (*c* 0.05, H_2O),[#] which was prepared from the resolved potassium salt.[7] The precipitated $BaSO_4$ was filtered off and the diastereoisomer (1.2 g) obtained by the addition of EtOH and Et_2O to the solution while cooling in ice.

(−)-2-*tert*-Butylamino-1-(4'-hydroxy-3'-hydroxymethylphenyl)-ethanol Hydrochloride Monohydrate. To the diastereoisomer (1.0 g) in H_2O (4 ml) was added $BaCl_2$ (0.22 g). The $(+)_{546}$-Ba[CoEDTA)$_2$·$4H_2O$ was recovered by the addition of EtOH and Et_2O while cooling in ice. (−)-Salbutamol was precipitated as the HCl salt from the oil formed on evaporation of the filtrate at reduced pressure. The recrystallized product yielded 0.24 g, $[\alpha]^{20}D$ −32.2° (*c* 0.10, H_2O). The compound changed crystalline form at 175° and decomposed over the range 185–195°. *Anal.* ($C_{13}H_{22}NO_3Cl$) C, H, N.

(+)-2-*tert*-Butylamino-1-(4'-hydroxy-3'-hydroxymethylphenyl)-ethanol Hydrochloride Monohydrate. A HCl salt of (+)-salbutamol was prepared from the oil obtained on reducing the volume of the filtrate remaining after diastereoisomer removal. The recrystallized product yielded 0.15 g, $[\alpha]^{20}D$ +30.8° (*c* 0.10, H_2O). The compound changed crystalline form at 175° and decomposed over the range 185–195°. *Anal.* ($C_{13}H_{22}NO_3Cl$) C, H, N.

Relaxation Studies. The drugs were tested by a cumulative dose method on guinea pig tracheal chain at a tension of 300 mg in Krebs physiological salt solution. Linear regression lines were obtained by a least-squares method. Mean log ED_{50} and the standard error of the mean were found for each drug and tested at the 10% significance level for differences between the drugs using a student's *t* test. The mean log dose-response curves were obtained from approximately 20 tissue experiments for each drug. The tissue responses were recorded on a Hewlett-Packard 680M recorder using a Sanborn FTA-1-1 microforce transducer with a Sanborn Model 311A amplifier.

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A Synthesis of Noformycin

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Noformycin (5) was isolated from a culture of *Nocardia formica* and was identified as the active constituent of this microorganism.[1-3] This material was unusual in that it exhibited a wide range of antimicrobial activity. Of particular interest was its *in vivo* activity in mice against swine influenza and SK poliomyelitis. Subsequent to its isolation and identification, noformycin was tested against a wide variety of plant and animal viruses[4-7] and found to possess very potent activity. However, this material appeared to possess considerable toxicity, which was confirmed in our laboratories.

In view of the broad spectrum of activity, we became interested in synthesizing homologs of noformycin with the expectation of reducing toxicity while retaining activity. Specifically, we were interested in developing a versatile synthesis which would adapt itself to a variety of transformations. A detailed synthesis of noformycin itself has not been published although it has been reported that the synthetic racemic material possesses half the activity of the isomer obtained from the culture.[2] Consequently, we wish to report a facile synthesis of both racemic and (+)-noformycin which

§ Kindly supplied by Allen and Hanburys Ltd., England.
# EDTA is ethylenediaminetetraacetate.

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STUDIES ON THE EFFECTS OF ENANTIOMERS OF SOTERENOL, TRIMETOQUINOL AND SALBUTAMOL ON *BETA* ADRENERGIC RECEPTORS OF ISOLATED GUINEA-PIG ATRIA AND TRACHEA[1]

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ABSTRACT

BUCKNER, C. K. AND P. ABEL: Studies on the effects of enantiomers of soterenol, trimetoquinol and salbutamol on *beta* adrenergic receptors of isolated guinea-pig atria and trachea. J. Pharmacol. Exp. Ther. 189: 615–625, 1974.

Beta adrenergic receptors of guinea-pig atria and trachea were investigated with enantiomers of tissue selective agonists: soterenol, trimetoquinol and salbutamol. If the receptors of these two tissues are different, the ratio of potencies between enantiomers of the agonists should reflect the unique asymmetries of the receptors. From atria and trachea, enantiomeric potency differences (in log units) are: for soterenol, 1.58 and >2.10; for trimetoquinol, 1.61 and 1.58 and for salbutamol, 1.56 and >2.19, respectively. From both tissues, the values for each pair of isomers are identical. Analysis of tissue selectivity from potency values reveals that (−)-isomers of soterenol, trimetoquinol and salbutamol are, respectively, 3.2-, 9- and 24-fold more potent in trachea than atria. However, when compared to (−)-isoproterenol on a relative potency basis in each tissue, only salbutamol is shown to have any degree of selectivity for trachea. When relative activities are compared from atria, soterenol and salbutamol appear as "partial agonists" while trimetoquinol produces about 90% of the maximum effect of isoproterenol. All agonists produce the same maximum effects in trachea. In atria, sotalol blocks the effects of the isomers of soterenol to a greater extent than it blocks the effects of isoproterenol and the isomers of trimetoquinol. It is suggested that factors other than ligand binding modes on the receptor could account for these observations. The data support previous suggestions that agonist binding sites on *beta* receptors of guinea-pig atria and trachea may be similar. Tissue selectivity of agonists may reflect different requirements for access to receptors or intrinsic activities between atria and trachea.

The hypothesis that there are at least two types of *beta* adrenergic receptors was advanced to explain different orders of potencies of agonists and antagonists observed between several tissues (Furchgott, 1967; Lands *et al.*, 1967a,b). The subclassifications of *beta* receptors have been supported by observations that some newly synthesized *beta* receptor agonists exhibit marked *in vivo* and *in vitro* tissue selectivity (Brittain *et al.*, 1970; Farmer *et al.*, 1970a,b). In general, the new tissue-selective agonists were shown to be more potent in relaxing bronchial and uterine smooth muscle than in producing positive

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chronotropic or inotropic effects. Responses in the different types of tissue have been attributed to activation of so-called "*beta*-2" and "*beta*-1" receptors, respectively.

Even though tissue selectivity is clinically desired, factors other than qualitative differences in binding sites on the receptors may contribute to the phenomenon. For example, emphasizing the importance of changes in physicochemical properties with chemical structure, Buckner and Patil (1971) demonstrated that, under proper experimental conditions, the stereochemical selectivity for interaction of enantiomers of catecholamine agonists and competitive antagonists with *beta* receptors of guinea-pig atria and trachea was similar. This procedure minimized the problem of different barriers for access to the receptors by comparing potencies of compounds with the same physicochemical properties. Additionally, similar isomeric potency differences for a given pair of enantiomers suggested that the ligand binding sites on *beta* receptors of these two tissues may actually be of a similar type.

An additional problem in analysis of selectivity arises from observations that some tissue-selective agonists do not produce the same maximum mechanical effects as catecholamines in heart tissue, but appear to be "full agonists" only in smooth muscle (Farmer *et al.*, 1970a; present data). Therefore, tissue selectivity exerted by *beta* receptor agonists is not necessarily related to affinity for the receptor site, but may involve the ability to produce a response.

The availability of resolved isomers of some newer, tissue-selective *beta* receptor agonists prompted us to investigate the effects of these compounds on guinea-pig atria and trachea, in light of the previous suggestion that observed tissue selectivity may not be due to different types of *beta* receptor binding sites.

Methods

Albino, female guinea pigs (O'Brien Farms, Madison, Wisc.), weighing 300 to 500 g, were killed by a sharp blow to the back of the head. The tissues were removed, trimmed of excess tissue and suspended in water-jacketed (37–38°C) 10-ml tissue baths containing a physiologic salt solution of the following composition: NaCl, 118 mM; KCl, 4.7 mM; $CaCl_2 \cdot 2H_2O$, 2.5 mM; $MgCl_2 \cdot 6H_2O$, 0.5 mM; $NaH_2PO_4 \cdot H_2O$, 1 mM; $NaHCO_3$, 25 mM; and glucose, 11 mM. The tissue baths and stock salt solution were aerated with a mixture of oxygen (95%) and carbon dioxide (5%). Mechanical responses were recorded on a Grass model 5 or model 79B polygraph *via* force-displacement transducers (FT-03).

Cumulative dose-response effects of the agonists were obtained by increasing the concentrations by a factor of about 3 while the previous dose remained in contact with the tissue (van Rossum, 1963). Each concentration was added only after the effects of the previous concentration reached maximum and remained constant. Final maximum responses were taken to be the effects occurring when a 3-fold increase in agonist concentration failed to further elicit a response. The time required to obtain complete dose-response effects varied with the agonist employed (see "Results"). All other compounds were added to the bath in a volume of 0.1 ml and allowed to interact with the tissue for fixed periods of time.

Isolated right atria. Spontaneous atrial contractions were recorded together with atrial rate which was monitored with Grass model 7-P4D tachographs to aid in determining when maximum responses occurred after a given concentration of agonist. The amount of tension exerted on each atrium was the maximum needed to obtain a pen deflection of about 0.5 cm/beat at the highest preamplifier sensitivity without recording background noise. Each tissue was allowed to equilibrate for 1 hour prior to addition of any drug and washings were made at 15-minute intervals during this period. For construction of dose-response curves, the initial rate (beats per minute) was taken as that occurring just prior to beginning cumulative drug addition.

Isolated tracheal strips. Trachea were cut in spiral fashion, each turn separated by 3 to 4 cartilage segments (Constantine, 1966). Each strip was approximately halved and each half mounted in a tissue bath. Resting tension was adjusted to 2 g and maintained at that level during equilibration and drug incubation periods. Strips were allowed to equilibrate for 2 hours prior to addition of any drug and washings were made at 15-minute intervals during this period. Relaxation produced by *beta* receptor agonists was studied after partial contraction with carbachol, 3×10^{-7} M. As previously determined, this concentration produces a degree of contraction representing approximately 30% of the maximum capable of being produced by this agonist. The contraction reaches maximum in 10 to 15 minutes and remains constant for at least 1 hour. In order to keep drug contact periods constant, cumulative addition of *beta* receptor agonists was begun 15 minutes after addition of carbachol to the bath.

Experimental protocol. Because of the long duration of action of most of the agonists examined, only one cumulative dose-response curve